6. Trinchieri, G. *et al. J. exp. Med.* **160**, 1147–1169 (1984).
7. Tigges, M. A., Casey, L. S. & Koshland, M. E. *Science* **243**, 781–786 (1989).
8. Takacs, L., Osawa, H. & Diamantstein, T. *Eur. J. Immun.* **14**, 1152–1156 (1984).
9. Jenkinson, E. J., Kingston, R. & Owen, J. J. T. *Nature* **329**, 160–162 (1987).
10. Tentori, L., Longo, D. L., Zuniga-Pflucker, J. C., Wing, C. & Kruisbeek, A. M. *J. exp. Med.* **168**, 1741–1747 (1988).
11. Plum, J. & de Smedt, M. *Eur. J. Immun.* **18**, 795–799 (1988).
12. MacDonald, H. R. *et al. Immunol. Rev.* **104**, 157–182 (1988).
13. Doetschman, T. *et al. Nature* **330**, 576–578 (1987).
14. Thomas, K. R. & Capecchi, M. R. *Cell* **51**, 503–512 (1987).
15. Thompson, S., Clarke, A. R., Pow, A. M., Hooper, M. L. & Melton, D. W. *Cell* **56**, 313–321 (1989).
16. Zijlstra, M., Li, E., Sajjadi, F., Subramani, S. & Jaenisch, R. *Nature* **342**, 435–438 (1989).
17. Fuse, A. *et al. Nucleic Acids Res.* **12**, 9323–9331 (1984).
18. Zurawski, S. M. & Zurawski, G. *EMBO J.* **7**, 1061–1069 (1988).
19. Evans, M. J. & Kaufman, M. H. *Nature* **292**, 154–156 (1981).
20. Shortman, K., Egerton, M., Spangrude, G. J. & Scollay, R. *Semin. Immun.* **2**, 3–12 (1990).
21. von Boehmer, H. & Kisielow, P. *Science* **248**, 1369–1372 (1990).
22. Weinberg, K. & Parkman, R. *New Engl. J. Med.* **322**, 1718–1723 (1990).
23. Chen-Bettecken, U., Wecker, E. & Schimpl, A. *Proc. natn. Acad. Sci. U.S.A.* **82**, 7384–7388 (1985).
24. Pahwa, R. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 5069–5073 (1989).
25. Hooper, M. *et al. Nature* **326**, 292–295 (1987).
26. Smith, A. G. & Hooper, M. L. *Devl. Biol.* **121**, 1–9 (1987).
27. Williams, R. L. *et al. Nature* **336**, 684–687 (1988).
28. Kubo, R., Born, W., Kappler, J., Marrack, P. & Pigeon, M. *J. Immun.* **142**, 2736–2742 (1989).
29. Molinaro, G. A., Maron, E., Eby, W. C. & Dray, S. *Eur. J. Immun.* **5**, 771–774 (1975).

ACKNOWLEDGEMENTS. We thank M. Hooper for ES cells, R. Swoboda for IL-2 DNA clones, H. Haber, R. Mitnacht and K. Borschert for assistance, and S. Siddell for critical reading of the manuscript. These studies were supported by the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie.

Making antibody fragments using phage display libraries

Tim Clackson*, Hennie R. Hoogenboom†, Andrew D. Griffiths† & Greg Winter*†

*MRC Laboratory of Molecular Biology and †Centre for Protein Engineering, Hills Road, Cambridge CB2 2QH, UK

To by-pass hybridoma technology and animal immunization, we are trying to build antibodies in bacteria by mimicking features of immune selection[1]. Recently we used fd phage[2] to display antibody fragments fused to a minor coat protein[3,4], allowing enrichment of phage with antigen[3]. Using a random combinatorial library of the rearranged heavy (VH) and kappa (Vκ) light chains[5–8] from mice immune to the hapten 2-phenyloxazol-5-one (phOx), we have now displayed diverse libraries of antibody fragments on the surface of fd phage. After a single pass over a hapten affinity column, fd phage with a range of phOx binding activities were detected, at least one with high affinity (dissociation constant, $K_d = 10^{-8}$ M). A second pass enriched for the strong binders at the expense of the weak. The binders were encoded by *V* genes similar to those found in anti-phOx hybridomas but in promiscuous combinations (where the same *V* gene is found with several different partners). By combining a promiscuous *V*H or *V*κ gene with diverse repertoires of partners to create hierarchical libraries, we elicited many more pairings with strong binding activities. Phage display offers new ways of making antibodies from *V*-gene libraries, altering V-domain pairings and selecting for antibodies with good affinities.

We used the polymerase chain reaction (PCR)[9] to amplify the *V*H and *V*κ genes from the spleen messenger RNA of mice immunized with phOx, and also developed a 'PCR assembly' process[10] to link these genes together randomly for expression as single-chain Fv (scFv) fragments[11,12] (Fig. 1*a–c*). The assembled genes were cloned in a single step into the vector fdDOG1 (Fig. 1*e*) for display as a fusion with the fd gene III coat protein. This initial library of 2×10^5 clones seemed to be diverse (Fig. 1*d*), and sequencing revealed the presence of most VH groups[13] and Vκ subgroups[14] (data not shown). None of the 568 clones tested bound to phOx as detected by enzyme-linked immunosorbent assay (ELISA).

The library of phages was passed down a phOx affinity column (Table 1*a*), and eluted with hapten. Of the eluted clones, 13%

TABLE 1 Affinity selection of hapten-binding phage

		Clones binding to phOx*		
	Precolumn	After first round	After second round	After third round
(*a*) Random combinatorial libraries				
phOx-immunized mice	0/568 (0%)	48/376 (13%)	175/188 (93%)	—
Unimmunized mice	—	—	0/388 (0%)	—
(*b*) Hierarchical libraries				
*V*H-B/*V*κ-*rep* library	6/190 (3%)	348/380 (92%)	—	—
*V*H-rep/*V*κ-d library	0/190 (0%)	23/380 (7%)	—	—
(*c*) Fractionation of VH-B/Vκ-d and VH-B/Vκ-b phage*				
Mixture of clones	88/1,896 (4.6%) (44/1,740 (2.5%)†)	55/95 (57.9%)	1,152/1,156 (99.7%)	1,296/1,299 (99.8%)

Selection of phage with hapten-binding activities from the random combinatorial and hierarchical libraries (*a* and *b*, respectively), and fractionation of clones with different affinities for phOx (*c*). For the random combinatorial libraries fdDOG1 RF was extensively digested with *Not*I and *Apa*LI, purified by electroelution[24] and 1 μg ligated to 0.5 μg (5 μg for the hierarchical libraries) of the assembled *scFv* genes in 1 ml with 8,000 units T4 DNA ligase (New England Biolabs) overnight at 16 °C. Purified ligation mix was electroporated in six aliquots into MC1061 cells[25] and plated on NZY medium[24] with 15 μg ml^{-1} tetracycline, in 243 × 243 mm dishes (Nunc); 90–95% of clones contained *scFv* genes by PCR screening (see legend to Fig. 1). Colonies were scraped into 50 ml 2 × TY medium[26] and shaken at 37 °C for 30 min. Liberated phage were precipitated twice with polyethylene glycol and resuspended to 10^{12} transducing units (TU) ml^{-1} in water (titred as in ref. 3). For affinity selection, a 1-ml column of phOx-BSA-Sepharose[27] M. Dreher and C. Milstein, unpublished results) was washed with 300 ml PBS, and 20 ml PBS containing 2% skimmed milk powder (MPBS). Phage (10^{12} TU) were loaded in 10 ml MPBS, washed with 10 ml MPBS and finally 200 ml PBS. The bound phage were eluted with 5 ml 1 mM 4-ε-amino-caproic acid methylene 2-phenyl-oxazol-5-one (phOx-CAP). About 10^6 TU eluted phage were amplified by infecting 1 ml log phase *E. coli* TG1 (ref. 28) and plating as above. For a further round of selection, colonies were scraped into 10 ml 2 × TY medium and then processed as above. For the hierarchical libraries, *V*H-B and *V*κ-d genes were individually recloned, then assembled with the *V*H or *V*κ repertoires. For the fractionation of clone VH-B/Vκ-d, 7×10^{10} TU phage in the ratio 20 VH-B/Vκ-b:1 VH-B/Vκ-d were loaded onto a phOx-BSA-Sepharose column in 10 ml MPBS and eluted as above. Eluted phage were used to reinfect *E. coli* TG1, and phage produced and harvested as before. About 10^{11} TU of phage were loaded onto a second affinity column and the process repeated to give a total of three column passes. Dilutions of eluted phage at each stage were plated in duplicate and probed separately[24] with oligonucleotides specific for *V*κ-b (5′-GAGCGGGTAACCACTGTACT) or Vκ-d (5′-GAATGGTATAGTACTACCCT).

* In (*c*), numbers refer to VH-B/Vκ-d.

† Numbers after three reinfections and cycles of growth. This control, omitting the column steps, confirms that a spurious growth or infectivity advantage was not responsible for the enrichment of clone VH-B/Vκ-d.

bound to phOx, and ranged from poor to strong binding in ELISA. We sequenced 23 of these hapten-binding clones and found eight different *V*H genes (A-H) in a variety of pairings with seven different *V*κ genes (a-g) (Fig. 2*a*). Most of the domains, such as VH-B and Vκ−d, were able to bind hapten with any of several partners[15]. The probability of finding multiple partners for a given chain should depend mainly on the inherent promiscuity of the chain and on the number of available partners and competing chains. Two other examples of promiscuous pairings have been noted in random combinatorial libraries made in λ phage[6,8], so this may prove to be a feature of small combinatorial libraries from immunized animals.

The sequences of the *V* genes were related to those seen in the secondary response to phOx, but with differences (Fig. 2*b*). Thus most phOx hybridomas from the secondary response use somatically mutated derivatives of three types of *V*κ genes, *V*κox1, '*V*κox-like' and *V*κ45.1 genes[16]. These can pair with *V*H genes from several groups, but *V*κox1 more commonly pairs with the *V*Hox1 gene (*V*H group 2; ref. 13). *V*κox1 genes are always, and *V*κox-like genes often, found in association with heavy chain genes (including *V*Hox1) that encode a short five-residue CDR3, with the sequence motif Asp-X-Gly-X-X (where X is any amino acid[16]), in which the central glycine creates a cavity for phOx (ref. 17). In our library, nearly all of the *V*H genes belonged to group 1, and most of the *V*κ genes were ox-like and associated with *V*H genes encoding a five-residue CDR3 motif Asp/Asn-X-Gly-X-X (Fig. 2*b*). *V*κox1 and *V*Hox1 were found only once (*V*κ−f and *V*H-E), and not in combination with each other: indeed *V*κ-f does not encode the Trp 91 involved in phOx binding[17] and was paired with a *V*H gene (*V*H-C) encoding a six-residue CDR3.

The promiscuity of the VH-B and Vκ-d domains prompted us to force further pairings, by assembling these genes with the entire repertoires of either *V*κ or *V*H genes from the same immunized mice. The resulting hierarchical libraries, (*V*H-B× *V*κ-*rep* and *V*H-*rep*× *V*κ-d), each with 4×10^7 members, were subjected to a round of selection and hapten-binding clones isolated (Table 1*b*). Most were strong binders by ELISA

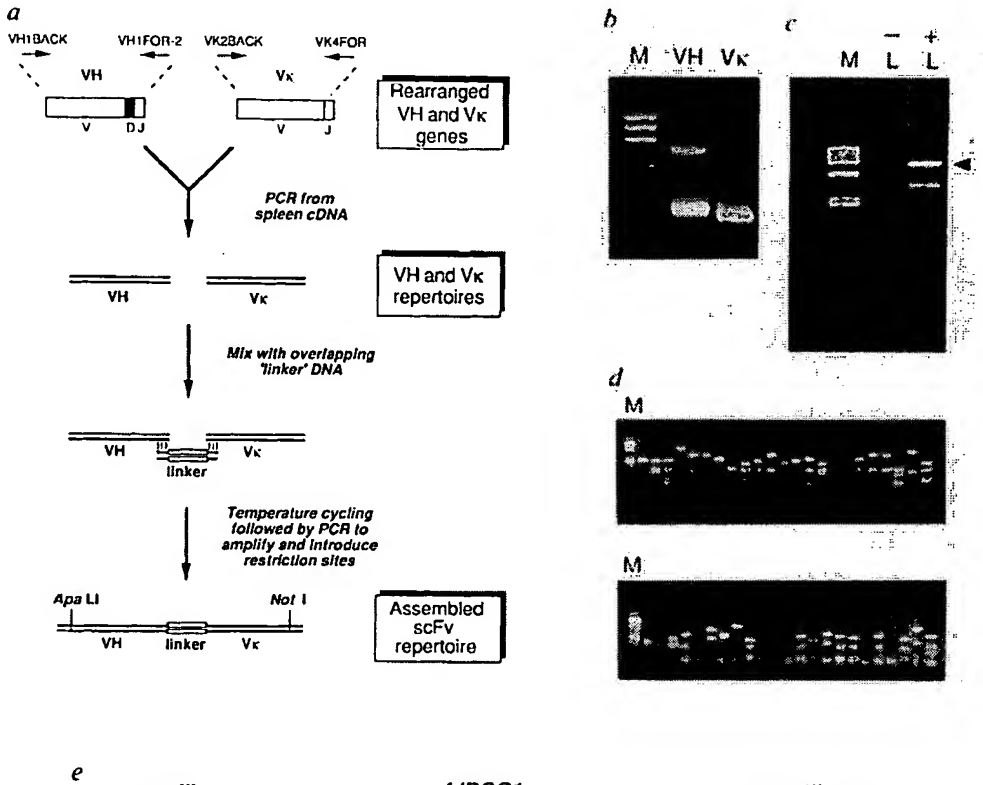


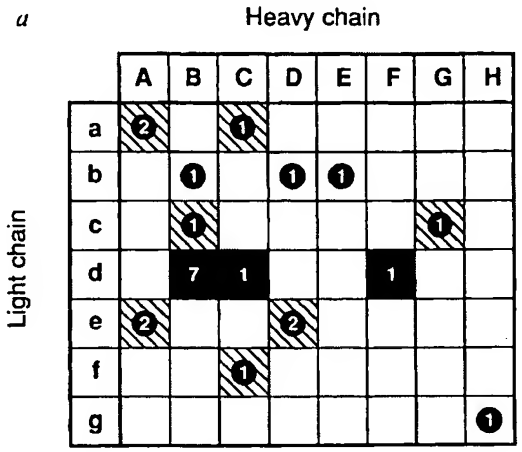
FIG. 1 PCR assembly of scFv library. *a*, *V*H and *V*κ genes are separately amplified, then mixed with a linker fragment that overlaps them both. The linker (93 base pairs) encodes the short peptide, $(Gly_4Ser)_3$, which links *V*H and *V*κ in scFvs (ref. 11). Cycles of annealing–denaturation, followed by reamplification of the mixture, generate a random combinatorial cassette of *V*H and *V*κ genes joined in-frame for expression. *b*, *V*H and *V*κ gene repertoire PCR products from the immunized mice analysed by electrophoresis on agarose (1%) gel. *c*, PCR assembly of *scFv* gene repertoires with linker (+L) or without (−L); arrow indicates assembled repertoire. M is DNA marker ΦX174 replicative form DNA digested with *Hae*III. *d*, Diversity of library as seen by *Bst*NI fingerprinting of individual clones. *e*, Sequence of *fd* gene III around the signal peptide cleavage site in fdDOG1.

METHODS. For the random combinatorial libraries, cytoplasmic RNA was isolated[29] from the pooled spleens of either 5 male BALB/c mice boosted 8 weeks after primary immunization with phOx coupled to chicken serum albumin[27], or of 5 unimmunized mice. The cDNA was made with avian myoblastosis virus reverse transcriptase (Anglian Biotech)[30] and primers that straddle the junction between the variable and constant regions of γ heavy chains and κ light chains (C. Marks, unpublished data). *V*H and *V*κ repertoires were amplified from the cDNA with 25 cycles of PCR (94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min) using Vent polymerase (New England Biolabs) and the primers VH1BACK (ref. 19) and VH1FOR-2 (ref. 31) or the primers VK2BACK and VK4FOR. The linker DNA was similarly amplified from pSW2scD1.3 (ref. 3) using primers LINKFOR and LINKBACK (complementary to VK2BACK and VH1FOR-2 respectively). After gel purification, 1 μg each of the *V*H and *V*κ products were mixed with 300 ng linker in a 25 μl PCR reaction mix without primers, and cycled 7 times (94 °C 2 min, 72 °C 4 min) to join the fragments, then amplified for 20 cycles (94 °C 1.5 min, 72 °C 2.5 min) using 25 pmol each VH1BACK and VK4FOR primers. Finally, the assembled products were gel-purified and reamplified with VH1BACK-ApaLI and VK4FOR-NotI ('tagged' versions of the original primers) to append restriction sites. Products (1–5 μg) were extensively digested with *Apa*LI and *Not*I for cloning into fdDOG1. Recombinant colonies were screened by PCR[32] with the primers VH1BACK and VK4FOR, followed by digestion with the frequently cutting enzyme *Bst*NI. Primers: VK2BACK 5′-GACATTGAGCTCACCCAGTCTCCA; VK4FOR, an equimolar mix of 5′-CCGTTTGATTTCCAGCTTGGTGCC, 5′-CCGTTTTATTTCCAGCTTGGTCCC, 5′-CCGTTTTATTTCCAACTTTGTCCC and 5′-CCGTTTCAGCTCCAGCTTGGTCCC; LINKFOR, 5′-TGGAGACTGGGTGAGCTCAATGTC; LINKBACK, 5′-GGGACCACGGTCACCGTCTCCTCA; VH1BACK-ApaLI, as VH1BACK (ref. 19) but with 5′-CATGACCACAGTGCAC added at the 5′ end; VK4FOR-NotI, as VK4FOR but with 5′-GAGTCATTCTGCGGCCGC similarly added (restriction sites underlined).

FIG. 2 *a*, Matrix of V_H and V_κ genes identified in phOx-binding clones selected from random combinatorial library. The number of clones found with each combination is shown. The binding to phOx–BSA, as judged by the ELISA signal, seemed to vary (marked by shading): no binding was seen to BSA alone. Optical density at 405 nm: 0.2–0.9, dotted box; 0.9–2.0, hatched box; >2.0, solid box. *b*, Encoded protein sequences of phOx-binding clones. Sequences of phOx-binding clones isolated (single-letter amino-acid code) after one round of selection of the random combinatorial library, with pairings as above, or the hierarchical library. Note that the first eight or seven residues, and the last nine or eleven residues, of the V_κ or V_H genes, respectively, are encoded by the PCR primers. Classifications into V_H groups[13] and V_κ subgroups[14], and the position of residue 91 encoded by the V_κ genes (#), are indicated. The relationship to genes from the hybridoma analysed secondary response to phOx (ref. 16) is also shown; all of the V_κ genes are 'ox-like', apart from those marked * with an asterisk, which are $V_\kappa$ox1, and the only example of V_H group 2 (V_H-E) is $V_H$ox1. The intensity of ELISA signals from the hierarchical libraries, corrected relative to the signal from control phage, are indicated: Optical density at 405 nm 0.9–2.0 (++), >2.0 (+++). Multiple isolations of sequences are marked, and sequences (V_H-B and V_κ-c) isolated from the random combinatorial library, and also the hierarchical libraries, are shown in italics. The V_H-B/V_κ-d and V_H-B/V_κ-c pairings gave similar signals (after correction of ELISA) when recovered from either combinatorial or hierarchical libraries.

METHODS. We screened for binding of the phage to hapten by ELISA: 96-well plates were coated with 10 $\mu g\ ml^{-1}$ phOx–BSA[27] or 10 $\mu g\ ml^{-1}$ BSA in PBS overnight at room temperature. Colonies of phage-transduced bacteria were inoculated into 200 μl 2×TY medium[26] with 12.5 $\mu g\ ml^{-1}$ tetracycline in 96-well plates ('cell wells', Nuclon) and grown with shaking (300 r.p.m.) for 24 h at 37 °C. At this stage, cultures were saturated and phage titres were reproducible (10^{10} TU ml^{-1}). Phage supernatant (50 μl), mixed with 50 μl PBS containing 4% skimmed milk powder, was then added to the coated plates. Further details given in ref. 3. To sequence the clones, template was prepared[24] from the supernatants of 10-ml cultures grown for 24 h, and sequenced using the dideoxy method[33] and a Sequenase kit (USB), with primer LINKFOR for the V_H genes and primer fdSEQ1 (5′-GAATTTTCTGTATGAGG) for the V_κ genes.

a



b

V_H sequences

From combinatorial library:

	CDR1	CDR2	CDR3	Group	ELISA signal
A	QVQLQQSGAELARPGASVKMSCKASGYTFT SYTMH WVKQRPGQGLEWIG YINPSSGYTNYNQKFKD KATLTADKSSSTAYMQLSSLTSEDSAVYYCAN RYGAY	WGQGTTVTVSS x4	1		
B	QVKLQQSGAELAKPGASVKMSCKASGYTFT RDWMH WLKQRPGQGLEWIG YINPSTGYTEYNQKFKD KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR NYGLY	WGQGTTVTVSS x9	1		
C	QVQLQQSGPELVKPGASVKMSCKASGYTFT SYVMH WVKQKPGQGLEWIG YINPYNDGTKYNEKFKG KATLTSDKSSSTAYMELSSLTSEDSAVYYCAI YRSFPY	WGQGTTVTVSS x3	1		
D	QVQLQQSGPELVKPGASVKISCKASGYSFT GYFMN WVKQSHGKSLEWIG RINPYNGDTFYNQKFKG KATLTVDKSSSTAHMELLSLTSEDSAVYYCVG ITTRFAY	WGQGTTVTVSS x3	1		(see Fig.2a)
E	QVQLQESGPGLVAPSQSLSITCTVSGFSLT SYGVH WVRQPPGKGLEWLG VIWAGGSTNYNSALMS	RLSISKDNSKSQVFLKMNSLQTDDTAMYYCAR DRGDY	WGQGTTVTVSS	2	Fig.2a)
F	QVQLQQSGPELAKPGASVKMSCKASGYTFT SYLNH WVKQRPGQGLKWIG YINPSTGYTEYNQKFKD KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR DYGYY	WGQGTTVTVSS	1		
G	QVKLQQSGAELVRPGASVKLSCKASGYTFT RYLNH WVKQRPGQGLEWIG YINPSTGYTEYNQKFKD EATLTADKSSNTAYMQLSSLTSEDSAVYYCAR DYGYY	WGQGTTVTVSS	1		
H	QVQLQQSGPELMKPGASVKISCKASGYSFS RNYMH WVKQSHGKSLEWIG YIAPFNGGTTYNQKFKG KATLTVDRSSSTAYMHLSSLTSEDSAVYYCAT DYGRD	WGQGTTVTVSS	1		

From hierarchical library V_H-rep×V_κ-d:

I	QVKLQQSGPELARPGVSVKMSCKASGYTFT SYAMH WVKQSQSKSLEWIG VISTYNGNTNYNQKFKG KATMTVDKSSSTAYMELARLTSEDSAIYYCAR DYGDY	WGQGTTVTVSS	1	+++	
J	QVKLQQSGAELARPGASVKMSCKASGYTFT RYTMH WVKQRPGQGLEWIG YINPSSGYTNYNQKFKD KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR DRGAY	WGQGTTVTVSS	1	+++	
K	QVKLQQSGAELAKPGASVKMSCKASGYTFT RDWMH WVKQRPGQGLEWIG YINPSTGYTEYNQKFKD KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR NYGLY	WGQGTTVTVSS x3	1	+++	
L	QVQLQQSGLELAKPGASVKMSCKASGYTFT NYLMH WVKQRPGQGLEWIG YINPSTGYTEYNQKFKD KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR DYGYY	WGQGTTVTVSS x2	1	++	
M	QVKLQQSGAELAKPGASVKMSCKASGYTFT NYWMH WVKQRPGQGLEWIG YINPSTGYTEYNQKFKD KATLTADKSSSTAYMQLSSLTSDDSAVYYCAR DYGYF	WGQGTTVTVSS	1	+++	
N	QVQLQQSGAELVKPGASVKLSCKTSGYTFT SYTMN WVKQRPGQGLEWIG YINPSSGYTNYNQKFKD KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR DYGYY	WGQGTTVTVSS	1	++	
O	QVQLQQSGAELAKPGASVKMSCEASGYTFT SHLMH WVKQRPGQGLEWIG YINPRTGYTEYNQKFKD KATFTADKSSSTAYMQLSSLTSEDSAVYYCAR DYGAY	WGQGTTVTVSS	1	++	
P	QVKLQQSGAELAKPGASVKMSCKASGYTFT SYWMH WVKQRPGQGLEWIG YINPSTGYTEYNQKFKD KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR DYGYY	WGQGTTVTVSS	1	++	
Q	QVQLQQSGAELAKPGASVKMSCKATGYTFT SYLMH WVKQRPGQGLEWIG YINPSTGYTEYNQKFKD KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR DYGYY	WGQGTTVTVSS	1	+++	
R	QVQLQQSGAELAKPGASVKMSCKASGYTFT SYVMH WVKQRPGQGLEWIG YINPSSGYTNYNQKFKD KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR NYGIY	WGQGTTVTVSS	1	++	
S	QVQLQQSGAELAKPGASVKMSCKASGYTFT TFLMH WLKQRPGQGLEWIG YINPSTGYTEYNQKFKD KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR DYGYY	WGQGTTVTVSS x2	1	+++	
T	QVKLQQSGAELARPGASVKMSCKASGYTFT SYTMH WVKQRPGQGLGWIG YINPSSGYTNYNQKFKD KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR DYGYY	WGQGTTVTVSS x6	1	+++	
U	QVKLQQSGAELAKPGASVKMSCKASGYTFT SYTMH WVKQRPGQGLEWIG YINPTTGYTEYNQKFKD KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR DYGYY	WGQGTTVTVSS	1	+++	
B	*QVKLQQSGAELAKPGASVKMSCKASGYTFT RDWMH WLKQRPGQGLEWIG YINPSTGYTEYNQKFKD KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR NYGLY*	*WGQGTTVTVSS*	1	+++	

V_κ sequences

From combinatorial library:

	CDR1	CDR2	CDR3			
a	DIELTQSPSSLSASLGERVSLTC RASQEISGYLS WLQQKPDGSIKRLIY AASTLES GVPKRFSGSRSGSDYSLTISSLESEDFADYYC LQYASYPT	FGAGTKLEIKRA x3	V			
b	DIELTQSPAIMSASPGEKVTMTC RASSSVSSSYLH WYQQKSGASPKVWIY STSNLAS GVPARFSGSGSGTSYSLTISSVEAEDAATYYC QQYSGYPLT	FGAGTKLEIKRA x3	IV			
c	DIELTQSPTTMAASPGEKITITC SASSSISSNYLH WYQQKPGFSPKLLIY RTSNLAS GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC QQGSSIPLT	FGAGTKLEIKRA x2	IV	(see		
d	DIELTQSPTTMAASPGEKITITC SASSSISSNYLH WFQQKPGFSPKLLIS RTSNLAS GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC QQGSTIPFT	FGSGTKLEIKRA x9	IV	Fig.2a)		
e	DIELTQSPAIMSASPGEKVTITC SASSSVNYMH WFQQKPGTSPKLWIY STSNLAS GVPTRFSGSGSGTSYSLTISRMEAEDAATYYC QQRSSYPPT	FGSGTKLEIKRA x4	VI			
f	DIELTQSPAIMSAFPGEKVTMTC SASSSVSYNN WYQQKSGTSPKRWIY DTSKLAS GVPARFSGSGSGTSYSLTISSMEAEDAATYYC QQFSSNPLT	FGAGTKLELKRA	VI	*		
g	DIELTQSPAIMSASPGEKVTMTC SASSSINYMH WYQQKPGASPKRWIY DTSKLAS GVPARFSGSGSGTSYSLTISSMEAEDAATYYC HQRNSYPWT	FGGGTKLEIKRA	VI			

From hierarchical library V_H-B×V_κ-rep:

h	DIELTQSPAIMSASPGEKVTMTC SASSSVSYMH WYQQKSGTSPKRWIY DTSKLAS GVPARFSGSGSGTSYSLTISSMEAEDAATYYC QQWSSNPLT	FGAGTKLEIKRA x4	VI	*	+++	
i	DIELTQSPAIMSASPGEKVTITC SASSSVSYIH WFQQKPGTSPKLWIY STSNLAS GVPARFSGSGSGTSYSLTISRMEAEDAATYYC QQYHSYPLT	FGAGTKLEIKRA	VI		+++	
j	DIELTQSPTTMAASPGEKITITC SASSSISSNYLH WFQQKPGFSPKLLIY RTSNLAS GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC QQGSSIPLT	FGGGTKLEIKRA	IV		++	
k	DIELTQSPTTMAASPGDMITITC SATSSISSNYLH WYQQKPGFSPKLLIY RTSNLAS GVPPRFSGSGSGTSYSLTIGAMEAEDVATYYC QQGSSIPYT	FGAGTKLEIKRA	IV		+++	
l	DIELTQSPTTMAASPGEKITITC SASSSISSNYLH WYQQKPGFSPKLLIY RTSNLAS GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC QQGSSIPYT	FGGGTKLEIKRA	IV		++	
m	DIELTQSPTTMAASPGEKITITC SASSSISSNHLH WYQQKPGFSPKLLIY RTSNLAS GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC QQGSGIPYT	FGGGTKLEIKRA	IV		+++	
n	DIELTQSPTTMAASPGEKITITC SASSSISSNYLH WYQQKPGFSPKLLIY RTSNLAS GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC QQGSSIPFT	FGGGTKLEIKRA	IV		++	
o	DIELTQSPAIMAASPGEKITITC SASSSISSNYLH WYQQKPGFSPKLLIY RTSNLAS GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC QQGSSIPYT	FGGGTKLEIKRA x2	IV		+++	
p	DIELTQSPAIMSASPGEKVTMTC SASSSVSYMH WYQQKSGTSPKRWIY DTSKLAS GVPARFSGSGSGTSYSLTISSMEAEDVATYYC QQWSSNPLT	FGAGTKLEIKRA x2	VI	*	+++	
q	DIELTQSPAIMSASPGDKVTLTC SASSSVRYVN WFQQKSGTSPKRWIY DTSKLAS GVPARFSGSGSGTSYSLTISSMEAEDAATYYC QQWTSNPPT	FGGGTKLEIKRA	VI	*	+++	
r	DIELTQSPAIMSASPGEKVTMTC SASSSVSYMH WYQQKSGTSPKRWIY DTSKLAS GVPARFSGSGSGTSYSLTISSMEAEDAATYYC QQWSTNALT	FGAGTKLEIKRA	VI	*	++	
s	DIELTQSPAIMSASPGEKVTMTC RASSSVTSSYLN WYQQKSGASPKLWVY STSNLAS GVPARFSGSGSGTSYSLTISSVEAEDAATYYC QQYSGYPLT	FGAGTKLEIKRA	IV		+++	
t	DIELTQSPAIMSASPGEKVTMTC RASSSVSSSYLN WYQQKSGASPKLWIY STSNLAS GVPARFSGSGSGTSYSLTISRMEAEDAATYYC QQRSSYPLT	FGAGTKLEIKRA	IV		+++	
u	DIELTQSPAIMSASPGEKVTMTC RASSSVSSSYLH WYQQKSGASPKLWIY STSNLAS GVPARFSGSGSGTSYSLTISSVEAEDAATYYC QQYSGYPLT	FGAGTKLEIKRA	IV		++	
v	DIELTQSPAIMSASPGEKVTMTC RASSSVSSSYLH WFQQKSGASPKLWIY STSNLPS GVPARFSGSGSGTSYSLTISSVEAEDAATYYC QQYSGYPLT	FGGGTKLEIKRA	IV		+++	
a	*DIELTQSPTTMAASPGEKITITC SASSSISSNYLH WYQQKPGFSPKLLIY RTSNLAS GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC QQGSSIPLT*	*FGAGTKLEIKRA*	*x3*	IV	++	

(Fig. 2*b*). By sequencing 23 clones from each library, we identified 14 new partners for VH-B and 13 for Vκ-d; apart from VH-B and Vκ-c, none of the previous VH-B or Vκ-d partners (or indeed other partners) cloned and sequenced from the random combinatorial library was isolated again. These features are consistent with the much larger number of available partners (4×10^7) for the VH-B (or Vκ-d) domain, and the promiscuous nature of the domain. The *Vκ* genes were mainly ox-like and the *VH* genes mainly group 1, but the only examples of *Vκox1* (Vκ-h, −p, −q and −r) encode Trp 91, and the VH-CDR3 motif Asp-X-Gly-X-X now predominates. Thus some features of the phOx hybridomas seem to emerge more strongly in the hierarchical library. The new partners differ from each other mainly by small alterations in the CDRs, indicating that much of the subtle diversity had not been tapped by the original random combinatorial library. More generally we find that a range of related antibodies can be made by keeping one of the partners fixed and varying the other, and this could be invaluable for fine tuning of antibody affinity and specificity.

To determine the range of antibody affinities for phOx, we recloned the combinations of VH-B with Vκ-b and Vκ-d (which gave weak and strong binding signals to phOx in ELISA) for secretion as soluble scFv fragments (Fig. 3, legend). Fluorescence quench titrations determined the K_d of VH-B/Vκ-d for phOx-GABA as 1.0×10^{-8} M (Fig. 3*a*), indicating that antibodies with affinities representative of the secondary response can be selected from phage display libraries. Indeed of anti-phOx hybridomas from the secondary response, only two (out of 11 characterized) secrete antibodies of a higher affinity than VH-B/Vκ-d (ref. 16). The K_d of VH-B/Vκ-b for phOx-GABA was determined as 1.8×10^{-5} M (Fig. 3*b*); thus phage bearing scFv fragments with weak affinities can also be selected with antigen, probably because of the avidity of the multiple antibody heads on the phage.

A second round of selection of the original, random combinatorial library from immune mice resulted in 93% of eluted clones binding phOx (Table 1*a*). Most of these clones were Vκ-d combinations, and bound strongly to phOx in ELISA (data not shown). Few weak binders were seen. This suggested that affinity chromatography had not only enriched for binders, but also for the best. To confirm this we mixed the phage VH-B/Vκ-d with a 20-fold excess of the phage VH-B/Vκ-b and subjected the mixture to rounds of selection: after only two rounds, essentially all the eluted phage were VH-B/Vκ-d (Table 1*c*).

We also constructed a random combinatorial library (2×10^6 members) from unimmunized mice, but found no phOx-binding clones after two rounds of selection (Table 1*a*). Immunization therefore seems to be necessary to create and/or enrich for VH or Vκ domains with at least some of the features required for hapten binding. With libraries of this size (~10^6 members), such domains need to be represented at a high frequency to reconstitute a binding site[1], and immunization ensures this by biasing the spleen lymphoid cell population heavily towards messenger RNA-rich blast cells making specific antibody (R. Hawkins and G.W., unpublished data). With larger libraries (>10^7) now accessible using selection[3] rather than screening[5–8], immunization may be unnecessary for the isolation of antibody fragments. It has been estimated that a library of 10^7 different antibodies will probably recognize >99% of epitopes with a dissociation constant of $\geq10^{-5}$ M (ref. 18), and we have shown here that we can recover antibody fragments with such affinities

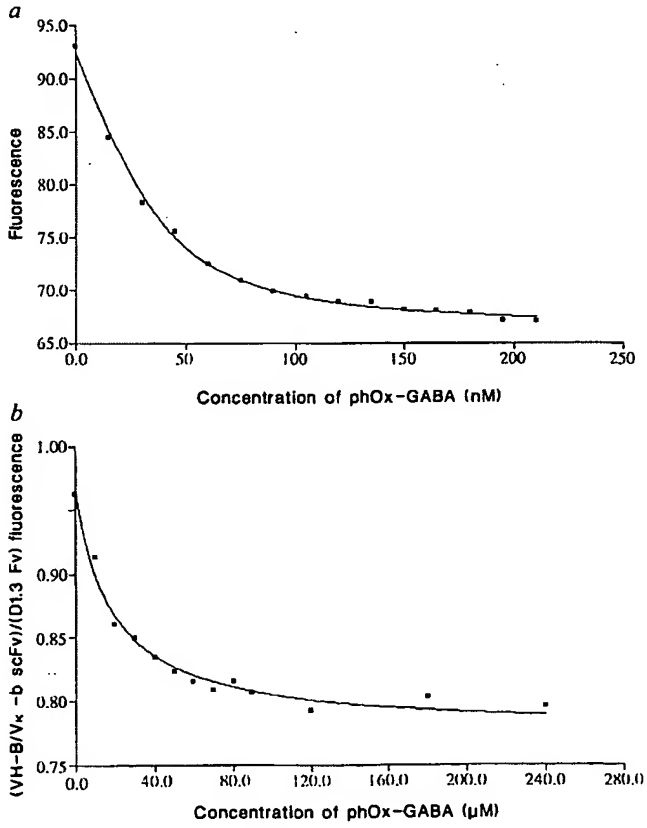


FIG. 3 Fluorescence quench titration of soluble scFv fragments. *a*, The K_d ($1.0\pm0.2\times10^{-8}$ M) for clone VH-B/Vκ-d was determined by fluorescence quench titration[34] of purified scFv (100 nM) with 4-γ-amino-butyric acid methylene 2-phenyl-oxazol-5-one (phOx-GABA). Excitation was at 280 nm, emission was monitored at 340 nm and the K_d calculated as in refs 35 and 36. All values were calculated with standard errors included. The K_d was determined to be $1.0\pm0.2\times10^{-8}$ M with 0.38 ± 0.05 binding sites per scFv molecule. *b*, For measurement of the K_d of the low affinity clone VH-B/Vκ-b, 2 μM purified scFv protein was titrated with phOx-GABA as above. But to minimize light absorption by the higher concentrations of phOx-GABA required, excitation was at 260 nm and emission was monitored at 304 nm. In addition, the fluorescence values were divided by those from a parallel titration of the lysozyme binding Fv fragment D1.3 (ref. 31). The K_d was calculated as described in refs 34 and 36 and determined to be $1.8\pm0.3\times10^{-5}$ M, with a fractional quench of 0.20 ± 0.01.

METHODS. Clones VH-B/Vκ-b and VH-B/Vκ-d were reamplified with VK4FOR-*NotI* and VH1BACK-*SfiI* (5′-CATGCCATGACTCGCGGCCCAGCCGGCCATGG-CC(G/C)AGGT(C/G)(A/C)A(A/G)CTGCAG(C/G)AGTC(A/T)GG-3′), a primer that introduces an *SfiI* site (underlined) at the 5′ end of the VH gene. *VH-B/Vk-d* was cloned into the phagemid pJM1 (A.D.G. and J. Marks, unpublished results) as an *SfiI–NotI* cassette, downstream of the pelB leader for periplasmic secretion[37], with a C-terminal peptide tag for detection[31,38], and under the control of a λP_L promoter[39]. Cultures (10 l) of *Escherichia coli* N4830-1 (ref. 40) harbouring each phagemid were induced[26] and supernatants precipitated with 50% ammonium sulphate. The resuspended precipitate was dialysed into PBS, 0.2 mM EDTA (PBSE), loaded onto a 1.5-ml column of phOx:Sepharose[41] and the column washed sequentially with 100 ml PBS; 100 ml 0.1 M Tris–HCl, 0.5 M NaCl pH 8.0; 10 ml 50 mM citrate, pH 5.0; 10 ml 50 mM citrate, pH4.0 and 20 ml 50 mM glycine, pH 3.0. The scFv fragment was eluted with 50 mM glycine, pH 2.0, neutralized with Tris base and dialysed against PBSE. VH-B/Vκ-b was cloned into a phagemid vector (A.D.G., unpublished results) based on pUC119 (ref. 42) encoding identical signal and tag sequences to pJM1, and expression induced at 30 °C in a 10-culture of *E. coli* TG1 (ref. 28) harbouring the phagemid, as in ref. 43. The low affinity of clone VH-B/Vκ-b made its purification on phOx-Sepharose impossible. Therefore after concentration by ultrafiltration (Filtron, Flowgen) the supernatant (100 ml of 600 ml) was loaded onto a 1-ml column of protein A-Sepharose coupled[44] to the monoclonal antibody 9E10 that recognizes the C-terminal peptide tag[31,38]. The column was washed with 200 ml PBS and 50 ml PBS, 0.5 M NaCl. The scFv fragment was eluted with 100 ml 0.2M glycine, pH 3.0, with neutralization and dialysis as before.

from phage display libraries. The antibody fragments could be rebuilt from their genes into complete antibodies, and expressed in myeloma cells if required, as described in ref. 19.

It may be possible to retain the original V_H/V_K pairings of the splenocytes, as in hybridoma technology. In principle, PCR assembly could be used to construct such 'natural' libraries, if the *V* genes from individual cells could be amplified and assembled in capsules. More immediately, affinity selection from combinatorial and hierarchical libraries promises an attractive route to high-affinity antibodies, in particular those from humans that are difficult to produce by hybridoma technology[1]. But the use of phage display libraries is not limited to antibodies: it offers a powerful and general method to change and refine the properties of any other protein[20] or peptides[21-23] that can be displayed on the phage surface. □

Received 19 March; accepted 9 July 1991.

1. Winter, G. & Milstein, C. *Nature* **349**, 293–299 (1991).
2. Smith, G. P. *Science* **228**, 1315–1317 (1985).
3. McCafferty, J., Griffiths, A. D., Winter, G. & Chiswell, D. J. *Nature* **348**, 552–554 (1990).
4. Hoogenboom, H. R. *et al. Nucleic Acids Res.* **19**, 4133–4137 (1991).
5. Huse, W. D. *et al. Science* **246**, 1275–1281 (1989).
6. Caton, A. J. & Koprowski, H. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6450–6454 (1990); correction, **88**, 1590 (1991).
7. Mullinax, R. L. *et al. Proc. natn. Acad. Sci. U.S.A.* **87**, 8095–8099 (1990).
8. Persson, M. A. A., Caothien, R. H. & Burton, D. R. *Proc. natn. Acad. Sci. U.S.A.* **88**, 2432–2436 (1991).
9. Saiki, R. K. *et al. Science* **239**, 487–491 (1988).
10. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. & Pease, L. R. *Gene* **77**, 61–68 (1989).
11. Huston, J. S. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 5879–5883 (1988).
12. Glockshuber, R., Malia, M., Pfitzinger, I. & Plückthun, A. *Biochemistry* **29**, 1362–1367 (1990).
13. Dildrop, R. *Immun. Today* **5**, 85–86 (1984).
14. Kabat, E. A., Wu, T. T., Reid-Miller, M., Perry, H. M. & Gottesman, K. S. *Sequences of Proteins of Immunological Interest* (US Department of Health and Human Services, US Government Printing Office, 1987).
15. Hudson, N. W., Mudgett-Hunter, M., Panka, D. J. & Margolies, M. N. *J. Immun.* **139**, 2715–2723 (1987).
16. Berek, C., Griffiths, G. M. & Milstein, C. *Nature* **316**, 412–418 (1985).
17. Alzari, P. M. *et al. EMBO J.* **9**, 3807–3814 (1990).
18. Perelson, A. S. *Immunol. Rev.* **110**, 5–33 (1989).
19. Orlandi, R., Güssow, D. H., Jones, P. T. & Winter, G. *Proc. natn. Acad. Sci. U.S.A.* **86**, 3833–3837 (1989).
20. Bass, S., Greene, R. & Wells, J. A. *Proteins* **8**, 309–314 (1990).
21. Cwirla, S. E., Peters, E. A., Barrett, R. W. & Dower, W. J. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6378–6382 (1990).
22. Devlin, J. J., Panganiban, L. C. & Devlin, P. E. *Science* **249**, 404–406 (1990).
23. Scott, J. K. & Smith, G. P. *Science* **249**, 386–390 (1990).
24. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1989).
25. Dower, W. J., Miller, J. F. & Ragsdale, C. W. *Nucleic Acids Res.* **16**, 6127–6145 (1988).
26. Nagai, K. & Thøgersen, H. C. *Meth. Enzym.* **153**, 461–481 (1987).
27. Mäkelä, O., Kaartinen, M., Pelkonen, J. L. T. & Karjalainen, K. *J. exp. Med.* **148**, 1644–1660 (1978).
28. Gibson, T. *thesis*, Univ. Cambridge (1984).
29. Gherardi, E., Pannell, R. & Milstein, C. *J. Immunol. Meth.* **126**, 61–68 (1990).
30. Clackson, T., Güssow, D. & Jones, P. T. in *PCR, a Practical Approach* (eds McPherson, M. J., Taylor G. R. & Quirke, P.) (IRL, Oxford, in the press).
31. Ward, E. S., Gussow, D., Griffiths, A. D., Jones, P. T. & Winter, G. *Nature* **341**, 544–546 (1989).
32. Güssow, D. & Clackson, T. *Nucleic Acids Res.* **17**, 4000 (1989).
33. Sanger, F., Nicklen, S. & Coulson, H. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).
34. Eisen, H. N. *Meth. med. Res.* **10**, 115–121 (1964).
35. Segal, I. *Enzyme Kinetics* 73–74 (Wiley-Interscience, New York, 1975).
36. Foote, J. & Milstein, C. *Nature* (in the press).
37. Better, M., Chang, C. P., Robinson, R. R. & Horwitz, A. H. *Science* **240**, 1041–1043 (1988).
38. Munro, S. & Pelham, H. R. B. *Cell* **46**, 291–300 (1986).
39. Shimatake, H. & Rosenberg, M. *Nature* **292**, 128–132 (1981).
40. Gottesman, M. E., Adhya, S. & Das, A. *J. molec. Biol.* **140**, 57–75 (1980).
41. McManus, S. & Riechmann, L. *Biochemistry* **30**, 5851–5857 (1991).
42. Vieira, J. & Messing, J. *Meth. Enzym.* **153**, 3–11 (1987).
43. De Bellis, D. & Schwartz, I. *Nucleic Acids Res.* **18**, 1311 (1990).
44. Harlow, E. & Lane, D. *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1988).

ACKNOWLEDGEMENTS. We thank J. McCafferty for help in constructing the vector fdDOG1; J. Marks for the vector pJM1; C. Marks for oligonucleotide primers for cDNA synthesis and for D1.3 Fv protein; E. Gherardi for spleen mRNA; J. Foote for help with fluorescence quench titrations; and M. Dreher, L. Riechmann, J. Jarvis, D. Chiswell, A. R. Fersht and C. Milstein for advice. Supported by the MRC, the D. Collen Research Foundation, Leuven, and the European Molecular Biology Organisation (H.R.H), and by the Louis Jeantet Foundation (A.D.G. and T.C.).

Phosphorylation-regulated Cl^- channel in CHO cells stably expressing the cystic fibrosis gene

Joseph A. Tabcharani*, Xiu-Bao Chang†, John R. Riordan† & John W. Hanrahan*‡

* Department of Physiology, McGill University, 3655 Drummond Street, Montréal, Québec H3G 1Y6, Canada
† Research Institute, Hospital for Sick Children, and University of Toronto, Toronto, Ontario M5G 1X8, Canada

A CYCLIC AMP-stimulated choride conductance appears when the cystic fibrosis gene is expressed in non-epithelial cells by infection with recombinant viruses[1,2]. Cyclic AMP-stimulated conductance in this system is mediated by the same ohmic, low-conductance Cl^- channel as in human secretory epithelia[2-4], but control of this channel by phosphorylation has not been directly demonstrated. Here we report the appearance of the low-conductance Cl^- channel in Chinese hamster ovary cells after stable transfection with the cystic fibrosis gene. The channel is regulated on-cell by membrane-permeant analogues of cAMP and off-cell by protein kinases A and C and by alkaline phosphatase. These results are further evidence that the cystic fibrosis transmembrane regulator is a Cl^- channel which can be activated by specific phosphorylation events and inactivated by dephosphorylation; they reveal an unsuspected synergism between converging kinase regulatory pathways.

The coding sequence of the cystic fibrosis transmembrane regulator (CFTR) was cloned behind the metallothionein promoter of a plasmid that also contained a mutant dihydrofolate reductase gene, driven by the simian virus 40 early promoter (Fig. 1*a*). Stably transformed colonies were selected with methotrexate after calcium phosphate transfection of Chinese hamster ovary (CHO)-K1 cells. CFTR-expressing variants were chosen for further study on the basis of their capacity to produce a protein of the same apparent size as that present in T84 cell membranes in western blots probed with monoclonal antibodies against CFTR (Fig. 1*b*). CFTR protein was localized in a highly enriched plasma membrane vesicle fraction. In variants containing nearly the same amount of CFTR as T84 cells, cAMP-regulated chloride permeability, as monitored by ^{125}I efflux, was indistinguishable from that in T84 cells (Fig. 1*c*).

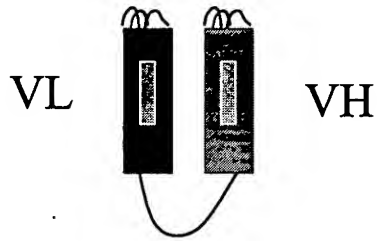
Patch-clamp recording was used to identify the channel responsible for cAMP-stimulated ^{125}I efflux. Channels became active in cell-attached patches after a lag of 69 ± 26 seconds when cells were exposed to membrane-permeant derivatives of cAMP; this was reversed by washing cAMP from the bath ($n = 7$, Fig. 2*a*). The channel was observed in 80% of all seals during cAMP stimulation (225/282) at an average density of between five and ten channels per patch. By contrast, it was recorded only once in 55 patches on unstimulated, CFTR-transfected cells and was never observed on cAMP-stimulated CHO cells that had been transfected with vector alone (0/31). Figure 2*b* shows that open probability was relatively independent of voltage, despite increased flickering at hyperpolarized potentials. Flickering was not observed using excised patches (see below), therefore these brief closures may reflect voltage-dependent, fast channel block by some anion in the cytosol. The current–voltage relationship rectified slightly in the outward direction during cell-attached recordings (Fig. 2*c*), but was linear ($r^2 = 0.9997$) when patches were excised and bathed symmetrically with 154 mM Cl^- (data not shown). In cell-attached patches the reversal potential (E_{rev}) was near the membrane potential (0.6 ± 0.3 mV applied potential) and the slope conductance at E_{rev} was 9.6 ± 0.5 pS ($n = 5$). The E_{rev} shifted to $+32.4 \pm 2.0$ mV when the pipette solution contained 110 mM sodium gluconate and

‡ To whom correspondence should be addressed.

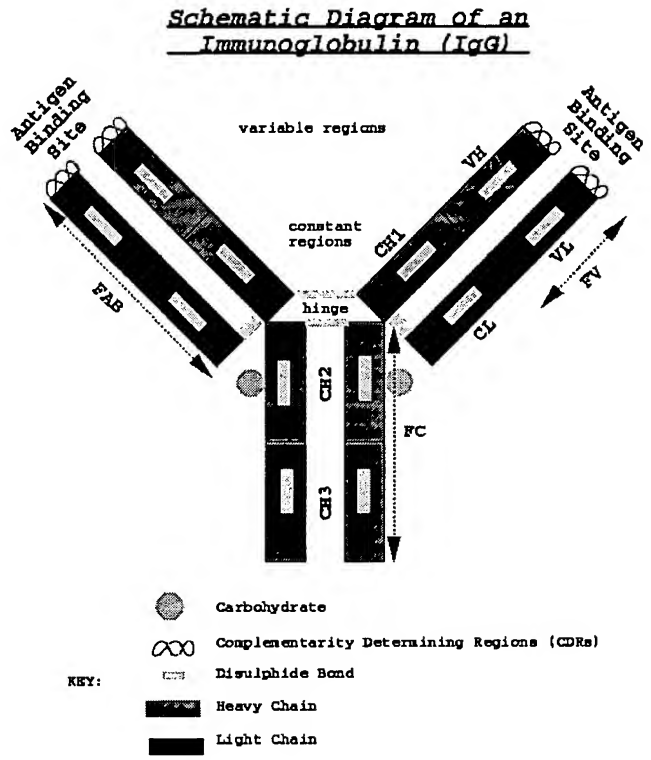
Enablement Issues in the Examination of Antibodies

Larry R. Helms
SPE, AU 1643
Technology Center 1600
USPTO
(571) 272-0832
Larry.helms@uspto.gov

Antibody Structure

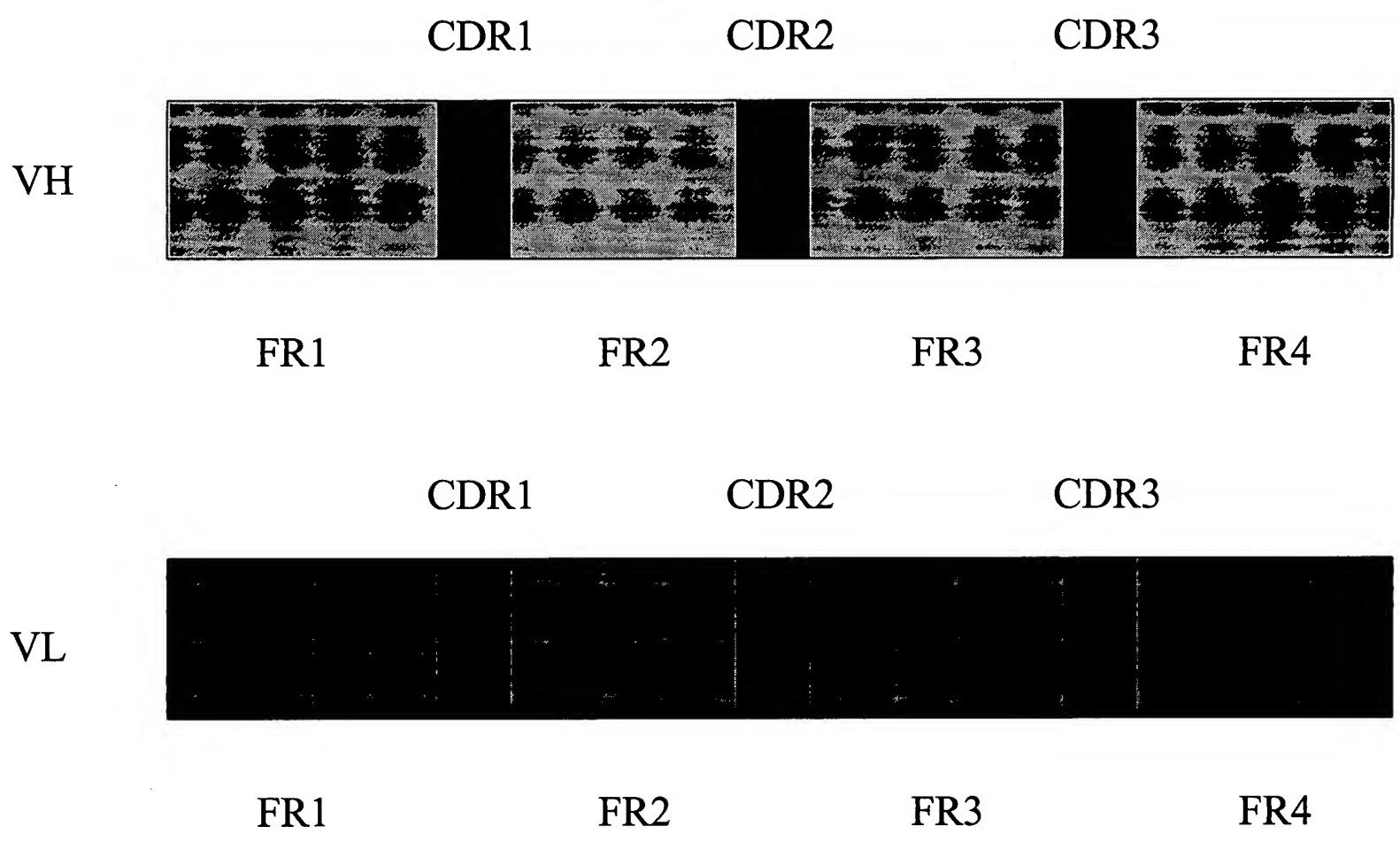


ScFv

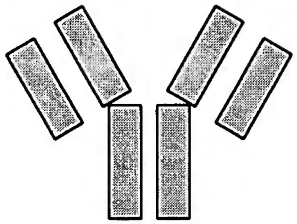


Adapted from people.cryst.bbk.ac.uk/~ubcg07s/gifs/igG.gif

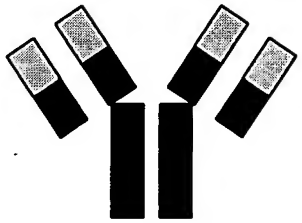
Variable domain of Antibodies



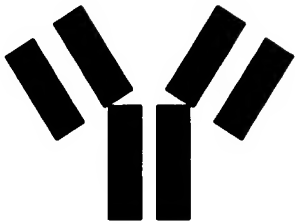
Humanization of Antibodies



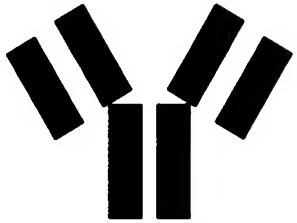
Mouse



Chimaeric



Humanized



Human

Enablement

35 USC § 112

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

MPEP 2164.01(a) Undue Experimentation Factors (*In re Wands*):

(1) The breadth of the claims

(2) The nature of the invention

(3) The state of the prior art

(4) The level of one of ordinary skill

(5) The level of predictability in the art

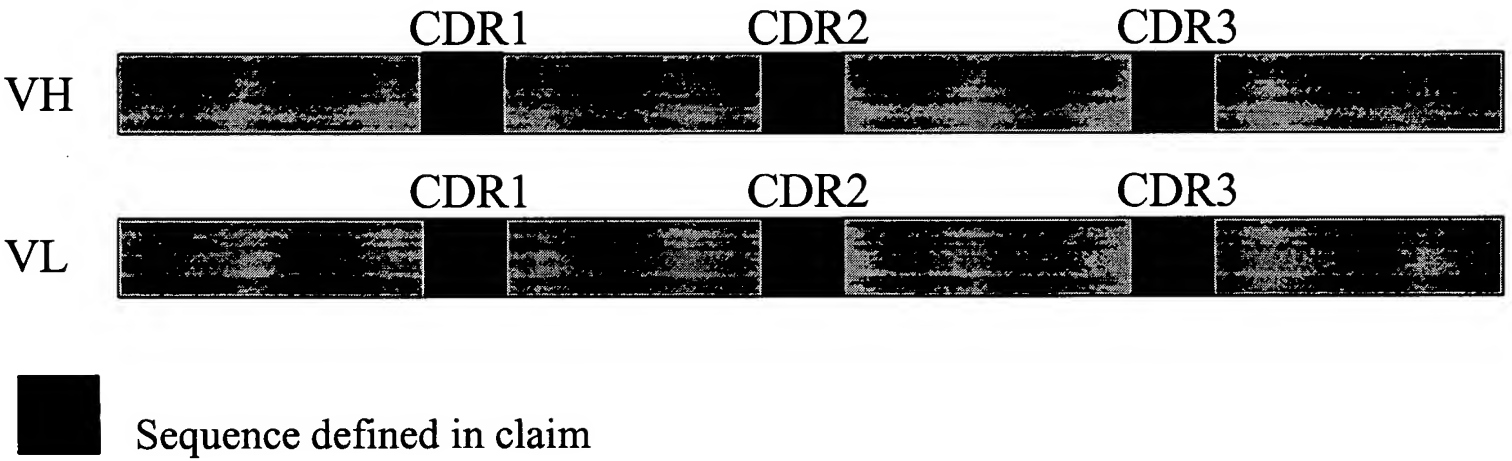
(6) The amount of direction provided by the inventor

(7) The existence of working examples

(8) The quantity of experimentation needed to make or use the invention based on the content of the disclosure

Example 1

- Claim: An isolated antibody that binds to human antigen X, said antibody comprises a heavy chain variable domain comprising the 3 CDRs in SEQ ID NO:1 and a light chain variable domain comprising the 3 CDRs in SEQ ID NO:2.



Specification

- Discloses antigen X from human tissue.
- Discloses antigen X is over-expressed in cancer tissue vs. normal tissue.
- The instant application produced an antibody that binds antigen X that contains a VH of SEQ ID NO:1 and a VL of SEQ ID NO:2, as well as explicitly disclosing humanized and chimaeric antibodies.
- The instant application provides examples of detection of cancer in human subjects with an antibody that binds antigen X.

State of the Prior Art

- It was well known at the time the application was filed that the heavy and light polypeptide chains each contribute three CDRs to the antigen binding region of the antibody molecule.
- The prior art[1] taught humanization of antibodies by transfer of the 6 CDRs from a donor framework region to an acceptor framework region and retention of antigen binding.

[1]Queen et al., PNAS (1988) 86:10029-10033,
Riechmann et al., Nature (1988) 332:323-327

Analysis

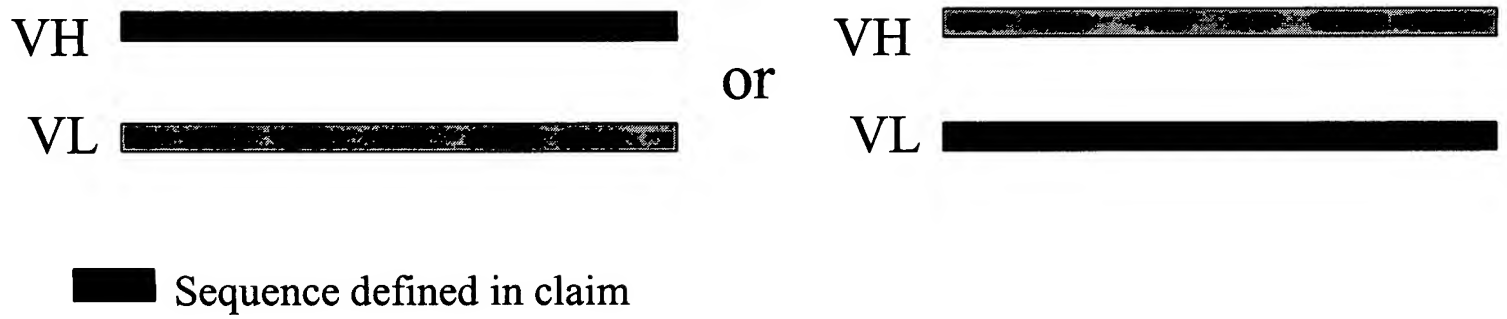
- In light of the prior art disclosing the CDRs as being the essential structure of the antibody's binding site, the identification of the specific CDR sequences in the specification provides enough structure to define the antibody's binding site.
- In addition, the prior art for humanization supports obtaining successful antigen binding by transferring the 6 CDRs from a donor framework to an acceptor framework.

Analysis (cont.)

- Thus, it would not have been undue experimentation to obtain an antibody that would bind antigen X and comprise the 6 CDRs as specifically defined in the claim at the time of filing.
- Therefore, a claim that defines an antibody that binds antigen X and comprises a heavy chain variable region comprising the 3 CDRs in SEQ ID NO:1 and a light chain variable region comprising the 3 CDRs in SEQ ID NO:2 meets the requirements under 35 U.S.C. 112, first paragraph, for enablement.

Example 2

- Claim 1. An isolated antibody that binds to human antigen X, said antibody comprises a heavy chain variable domain comprising SEQ ID NO:1.
- Claim 2. An isolated antibody that binds to human antigen X, said antibody comprises a light chain variable domain comprising SEQ ID NO:2.

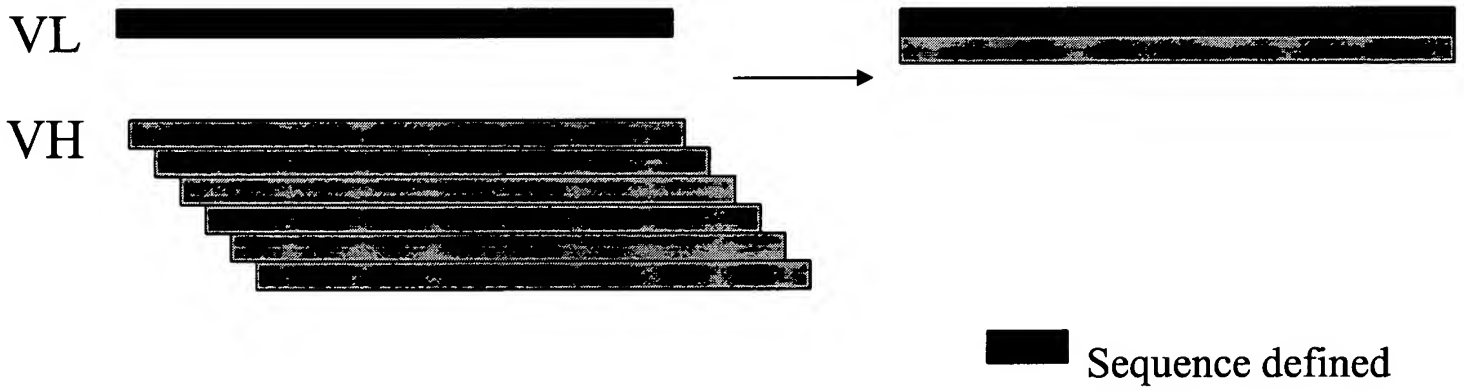


Specification

- Discloses antigen X from human tissue.
- Discloses antigen X is over-expressed in cancer tissue vs. normal tissue.
- The instant application produced an antibody that binds antigen X that contains a VH of SEQ ID NO:1 and a VL of SEQ ID NO:2, as well as explicitly disclosing humanized and chimaeric antibodies.
- The instant application provides examples of detection of cancer in human subjects with an antibody that binds antigen X.

State of the Prior Art

- There are several prior art[2] references that teach methods of producing antibodies that bind a specific antigen by using a specific VL (or VH) and screening a library of the complimentary variable domains.



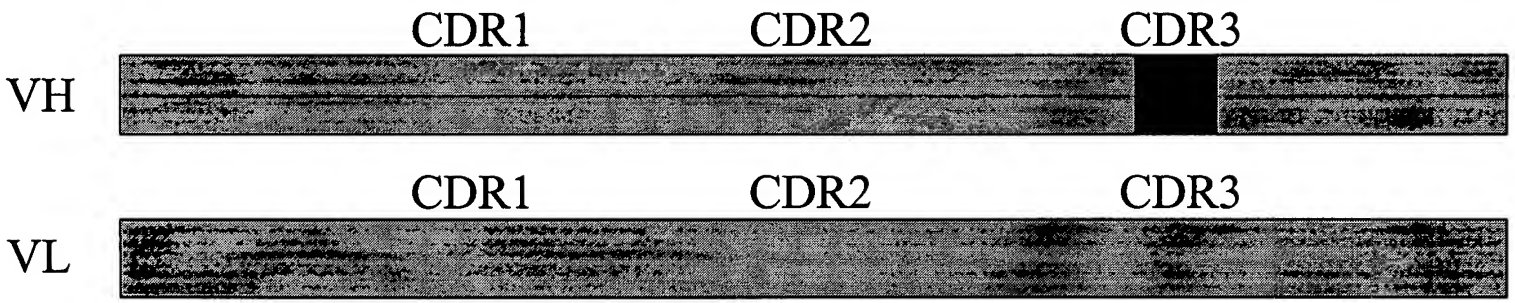
[2]Portolano et al., The Journal of Immunology (1993) 150:880-887
Clarkson et al., Nature (1991) 352:624-628

Analysis

- In light of the prior art disclosing methods of obtaining antibodies that bind an antigen by screening complementary variable domain libraries, the specification's disclosure of an antibody that binds a specific antigen comprising a defined VH or VL sequence would provide enough structure for one skilled in the art to practice the invention.
- Therefore, claims directed to an antibody that binds a specific antigen and comprises a defined VH or VL sequence meet the requirements under 35 U.S.C. 112, first paragraph, for enablement.

Example 3

- Claim: An isolated antibody that binds to human antigen X, said antibody comprises a heavy chain variable domain and a light chain variable domain, said heavy chain variable domain comprises the CDR3 in SEQ ID NO:1 (VH).



Sequence defined in claim

Specification

- Produced a series of antibodies that bind antigen X and the antibodies were not random combinations of VH and, i.e., VL they had specific VH domains paired with specific VL domains.
- The VH domains are highly homologous to each other and share not only CDR3, but also were nearly identical in framework regions (3-6/124 residues) as well as CDR1 (3/5)[1] and CDR2 (6/16)[1] regions.

| ▌indicates region where residues differ

[1] indicates residues that are identical out of number of residues in the CDR

Specification (cont.)

- Analysis of the VL sequences of these antibodies reveals that these domains are highly homologous to each other. The framework regions are nearly identical and the VL domains are identical in CDR1 and CDR2 regions. The CDR3 (8/10)[1] regions are highly homologous to each other.
- The instant application suggests that it was well established in the art at the time the invention was made that the CDR3 region alone can determine the specificity of the antibody.

[redacted]

[1] indicates residues that are identical out of number of residues in the CDR

State of the Prior Art

- Prior art for obtaining an antibody with only CDR3 of the VH defined:

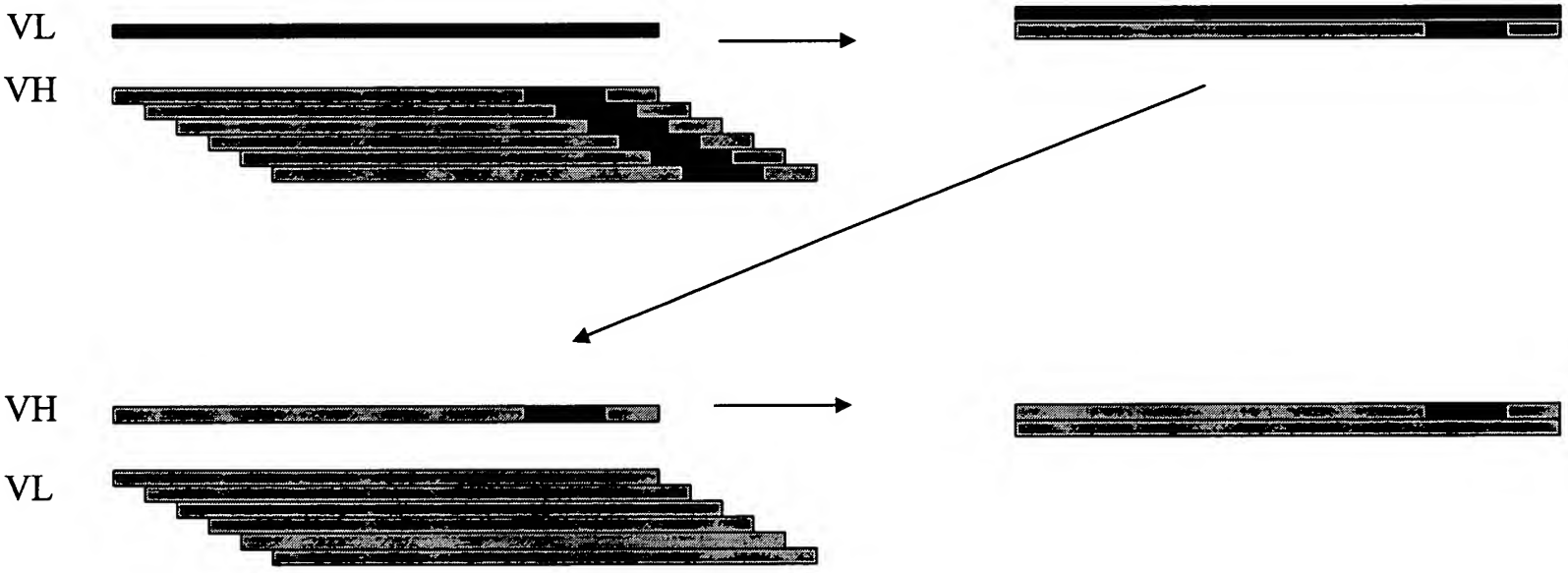
Klimka et al., British Journal of Cancer (2000) 83: 252-260: Klimka et al describe a screening process using a mouse VL and a human VH library with CDR3 and FR4 retained from the mouse VH. After obtaining antibodies, the VH was screened against a human VL library to obtain antibodies that bound antigen.

Beiboer et al., J. Mol. Biol. (2000) 296:833-849: Beiboer et al describe a screening process using the entire mouse heavy chain and a human light chain library. After obtaining antibodies, one VL was combined with a human VH library with the CDR3 of the mouse retained. Antibodies capable of binding antigen were obtained.

Rader et al., PNAS (1998) 95:8910-8915: Rader et al, describe a process similar to Beiboer et al above.

State of the Prior Art (cont.)

Method for screening



State of the Prior Art (cont.)

- The prior art methods for screening rely on a two step process where each step results in an antibody, however, each step requires one of the variable domains to be a defined sequence and the defined variable domain provides enough structure to obtain an antibody.
- The prior art methods do not result in an antibody solely by keeping CDR3 in the VH defined and randomizing the rest of the VH and VL domains.

State of the Prior Art (cont.)

- Prior art indicating the CDR3 region in the VH domain is important in antigen binding:

MacCallum et al., J. Mol. Biol. (1996) 262: 732-745: Analyzed many different antibodies for interaction with antigen and found that although CDR3 of the VH dominate the interaction, a number of residues outside the CDRs make antigen contacts and residues in the CDRs are important for backbone conformations.

Pascalis et al., the Journal of Immunology (2002) 169: 3076-3084: Grafting of CDRs onto a human framework required some residues in all 6 CDRs as well as specific frameworks.

Casset et al., BBRC (2003) 307, 198-205: Constructed a peptide mimetic of an anti-CD4 antibody binding site using 24 residues formed from residues from 5 of the CDRs. Casset et al., state that although CDR H3 is at the center of most antigen interactions, clearly other CDRs play an important role in recognition.

State of the Prior Art (cont.)

Vajdos et al., J. Mol. Biol. (2002) 320: 415-428: Antigen binding is primarily mediated by the CDRs but more highly conserved framework segments are mainly involved in supporting CDR loop conformations and, in some cases, framework residues also contact antigen.

Padlan et al., PNAS (1989) 86:5938-5942: Padlan et al describe the crystal structure of an antibody-lysozyme complex where all 6 CDRs contribute at least one residue to binding and one residue in the framework is also in contact with antigen.

Lamminmaki et al., JBC (2001) 276:36687-36694: Lamminmaki et al describe the crystal structure of an anti-estradiol antibody in complex with estradiol where, although CDRH3 plays a prominent role, all CDRs in the light chain make direct contact with antigen (even CDRL2, which is rarely directly involved in hapten binding).

State of the Prior Art (cont.)

- The prior art indicated that, in some instances, the CDR3 region is important. However, this region is not solely responsible for binding. The conformation of other CDRs, as well as framework residues influence binding.

State of the Prior Art (cont.)

- Transfer of only CDR3 in the VH and retention of antigen binding.

Barbas et al., PNAS (1995) 92: 2529-2533: Transferred the CDR3 of the VH of three anti-DNA antibody to an anti-tetanus toxoid antibody and retained DNA binding in 2/3 antibodies.

It was known in the art that antibodies that bind dsDNA can be generated by reconstruction of the CDR3 in the heavy chain of an antibody as well as transplantation of a 17 amino acid alpha-helical DNA binding domain into CDR3 of the heavy chain[3].

[3]McLane et al., PNAS (1995) 92:5214-5218,
Barbas et al., J. Am. Chem. Soc. (1994) 116:2161-2162

Analysis

- The claim is broadly drawn to any antibody that binds antigen X and comprises a heavy chain variable region comprising CDR3 in SEQ ID NO:1.
- The specification discloses antibodies with highly homologous VH and VL domains and identical VH CDR3 regions.
- The specification does not disclose that CDR3 of the VH alone can be transferred to just any framework and paired with just any VL and retain antigen binding.

Analysis (cont.)

- The specification does not provide any examples to support that CDR3 of the VH or VL is solely responsible for antigen binding.
- The prior art does not show screening for antibodies by just defining CDR3. The methods rely on using an entire VH or VL and screening random complimentary chains.
- The prior art does not show that a CDR3 is universally solely responsible for antigen binding.

Analysis (cont.)

- The prior art does not support a definition of an antibody structure solely by defining the CDR3 sequence of a VH or VL.
- Based on this analysis a claim to an isolated antibody that binds to human antigen X, said antibody comprises a heavy chain variable domain and a light chain variable domain, said heavy chain variable domain comprises the CDR3 in SEQ ID NO:1, does not meet the requirements of 35 U.S.C. 112, first paragraph, for enablement.

Questions

Larry R. Helms
SPE, AU 1643
Technology Center 1600
USPTO
(571) 272-0832
Larry.helms@uspto.gov

Yvonne (Bonnie) Eyler
Quality Assurance Specialist
Technology Center 1600
USPTO
(571) 272-0871
Yvonne.eyler@uspto.gov